FINAL REPORT

Study Title

In Vitro Mammalian Cell Gene Mutation Test
(L5178Y/TK$^{+/-}$ Mouse Lymphoma Assay)

Test Article

Ammonium perchlorate

Authors

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Study Completion Date

January 27, 1999

Performing Laboratory

BioReliance
9630 Medical Center Drive
Rockville, MD 20850

Laboratory Study Number

G98BA06.702

Sponsor

Perchlorate Study Group
Highway 50 and Aerojet Road
Building 20019/Department 0330
Rancho Cordova, CA 95813-6000

STATEMENT OF COMPLIANCE

Study G98BA06.702 was conducted in compliance with the US FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the US EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Regulations, the Japanese GLP Regulations and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test or control article have not been determined by the testing facility.

Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.

The stability of the test or control article under the test conditions has not been determined by the testing facility.

_____ _____

Richard H. C. San, Ph.D. Date
Study Director

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In Vitro Mammalian Cell Gene Mutation Test
(L5178Y/TK$^{+/-}$ Mouse Lymphoma Assay)

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Sponsor:	**Perchlorate Study Group** **Highway 50 and Aerojet Road** **Building 20019/Department 0330** **Rancho Cordova, CA 95813-6000**
Study Monitor:	**Michael F. Girard** **Perchlorate Study Group Representative**
Scientific Advisor:	**Michael L. Dourson, Ph.D., D.A.B.T.** **Toxicology Excellence for Risk Assessment**
Performing Laboratory:	**BioReliance** **9630 Medical Center Drive** **Rockville, MD 20850**
Test Article I.D.:	**ammonium perchlorate**
Test Article Lot No.:	**05006CQ**
Test Article Purity:	**99.999% (Provided by Sponsor)**
BioReliance Study No.:	**G98BA06.702**
Test Article Description:	**white, crystalline solid**
Storage Conditions:	**room temperature; protected from light and moisture**
Test Article Receipt:	**November 16, 1998**
Study Initiation:	**December 2, 1998**
Laboratory Manager:	**Jane J. Clarke, B.A.**

Study Director: _____

Richard H. C. San, Ph.D. Date

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SUMMARY

The test article, ammonium perchlorate, was tested in the L5178Y/TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay in the absence and presence of Aroclor-induced rat liver S9. The preliminary toxicity assay was used to establish the dose range for the mutagenesis assay. The mutagenesis assay was used to evaluate the mutagenic potential of the test article.

Dimethyl sulfoxide (DMSO) was selected as the solvent of choice based on solubility of the test article and compatibility with the target cells. The test article was soluble in DMSO at 500 mg/mL, the maximum concentration tested.

In the preliminary toxicity assay, the maximum concentration of ammonium perchlorate in treatment medium was 5000 μg/mL. No visible precipitate was present at any concentration in treatment medium. Selection of dose levels for the mutation assay was based on reduction of suspension growth relative to the solvent control. Substantial toxicity, i.e., suspension growth of ≤50% of the solvent control, was not observed at any concentration with or without S9 activation.

Based on the results of the preliminary toxicity assay, the doses chosen for the mutagenesis assay ranged from 50 to 5000 μg/mL for both the non-activated and S9-activated cultures. No visible precipitate was present at any concentration in treatment medium. No cloned cultures exhibited mutant frequencies that were at least 55 mutants per 10^6 clonable cells over that of the solvent control. There was not a dose-response trend. Toxicity in the cloned cultures, i.e., total growth of ≤50% of the solvent control, was not observed at any doses without activation but was observed with S9 activation at doses of 4000 and 5000 μg/mL.

The trifluorothymidine-resistant colonies for the positive and solvent control cultures were sized according to diameter over a range from approximately 0.2 to 1.1 mm. The colony sizing for the MMS positive control yielded the expected increase in small colonies, verifying the adequacy of the methods used to detect small colony mutants.

Under the conditions of this study, test article ammonium perchlorate was concluded to be negative in the L5178Y/TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay.

PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test article based on quantitation of forward mutations at the thymidine kinase locus of L5178Y mouse lymphoma cells.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, ammonium perchlorate, was received by BioReliance on November 16, 1998 and was assigned the code number 98BA06. The test article was characterized by the manufacturer as a white powder, which should be stored in a cool dry place. Its purity was given as 99.999%. Upon receipt, the test article was described as a white, crystalline solid and was stored at room temperature, protected from light and moisture.

The vehicle (solvent) used to deliver ammonium perchlorate to the test system was DMSO (CAS 67-68-5) obtained from Fisher.

Methyl methanesulfonate (MMS), CAS 66-27-3, lot # 09419LR, expiration date 5/01, was supplied by Aldrich Chemical Company and was used as the positive control for the non-activated test system at stock concentrations of 1000 and 2000 µg/mL. 7,12-Dimethyl-benz(a)anthracene (7,12-DMBA), CAS 57-97-6, lot # 85H0296, expiration date 1/99, was supplied by Sigma Chemical Company and was used at stock concentrations of 250 and 400 µg/mL as the positive control for the S9-activated test system.

MATERIALS AND METHODS

Test System

L5178Y cells, clone 3.7.2C, were obtained from Patricia Poorman-Allen, Glaxo Wellcome Inc., Research Triangle Park, NC. Each lot of cryopreserved cells was tested using the agar culture and Hoechst staining procedures and found to be free of mycoplasma contamination. Prior to use in the assay, L5178Y cells were cleansed of spontaneous $TK^{-/-}$ cells by culturing in a restrictive medium (Clive and Spector, 1975).

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor-1254, 500 mg/kg, five days prior to sacrifice. The S9 was batch prepared and stored at ≤-70°C until used. Each bulk preparation of S9 was assayed for sterility and its ability to metabolize 2-aminoanthracene and 7,12-dimethyl-benz(a)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S9 was mixed with the cofactors and Fischer's Medium for Leukemic Cells of Mice with 0.1% Pluronics (F_0P) to contain 250 µL S9, 6.0 mg nicotinamide adenine dinucleotide phosphate (NADP), 11.25 mg DL-isocitric acid and 750 µL F_0P per mL of S9-activation mixture and kept on ice until used. The cofactor/F_0P mixture was filter sterilized and adjusted to pH 7.0 prior to the addition of S9. The formulation of the activation mixture is based on information from Turner *et al.* (1984). The final concentration of S9 in the treatment medium was 10%.

Solubility Test

A solubility test was conducted to select the solvent. The test was conducted using one or more of the following solvents in the order of preference as listed: distilled water, dimethyl sulfoxide, ethanol and acetone. The test article was tested to determine the solvent, selected in order of preference, that permitted preparation of the highest soluble or workable concentration, up to 500 mg/mL (the highest concentration tested).

Preliminary Toxicity Assay

The preliminary toxicity assay was used to establish the optimal dose levels for the mutagenesis assay. L5178Y cells were exposed to the solvent alone and nine concentrations of test article ranging from 0.5 to 5000 µg/mL in both the absence and presence of S9-activation.

Cell population density was determined 24 and 48 hours after the initial exposure to the test article. The cultures were adjusted to $3x10^5$ cells/mL after 24 hours only. Cultures with less than $3x10^5$ cells/mL were not adjusted. Toxicity was measured as suspension growth relative to the growth of the solvent controls.

Mutagenesis Assay

The mutagenesis assay was used to evaluate the mutagenic potential of the test article. L5178Y mouse lymphoma cells were exposed to the solvent alone and at least eight concentrations of test article in duplicate in both the absence and presence of S9. Positive controls, with and without S9-activation, were tested concurrently.

Treatment of the Target Cells

The mutagenesis assay was performed according to a protocol described by Clive and Spector (1975). Treatment was carried out in conical tubes by combining 6 x 106 L5178Y/TK+/- cells, 4 mL F0P medium or S9 activation mixture and 100 µL dosing solution of test or control article in solvent or solvent alone in a total volume of 10 mL. A total of at least eight concentrations of test article were tested in duplicate. The positive controls were treated with MMS (at final concentrations in treatment medium of 10 and 20 µg/mL) and 7,12-DMBA (at final concentrations in treatment medium of 2.5 and 4.0 µg/mL). Treatment tubes were gassed with 5±1% CO2 in air, capped tightly, and incubated with mechanical mixing for 4 hours at 37±1°C. The preparation and addition of the test article dosing solutions were carried out under amber lighting and the cells were incubated in the dark during the exposure period. After the

treatment period, the cells were washed twice with F0P or F0P supplemented with 10% horse serum and 2 mM L-glutamine (F10P). After the second wash, the cells were resuspended in F10P, gassed with 5±1% CO2 in air and placed on the roller drum apparatus at 37±1°C.

Expression of the Mutant Phenotype

For expression of the mutant phenotype, the cultures were counted using an electronic cell counter and adjusted to $3x10^5$ cells/mL at approximately 24 and 48 hours after treatment in 20 and 10 mL total volume, respectively. Cultures with less than $3x10^5$ cells/mL were not adjusted.

For expression of the $TK^{-/-}$ cells, cells were placed in cloning medium (C.M.) containing 0.23% granulated agar. Two flasks per culture to be cloned were labeled with the test article concentration, activation condition, and either TFT (trifluorothymidine, the selective agent) or V.C. (viable count). Each flask was prewarmed to 37±1°C, filled with 100 mL C.M., and placed in an incubator shaker at 37±1°C until used. The cells were centrifuged at 1000 rpm for 10 minutes and the supernatant was decanted. The cells were then diluted in C.M. to concentrations of $3x10^6$ cells/100 mL C.M. for the TFT flask and 600 cells/100 mL C.M. for the V.C. flask. After the dilution, 1.0 mL of stock solution of TFT was added to the TFT flask (final concentration of 3 μg/mL) and both this flask and the V.C. flask were placed on the shaker at 125 rpm and 37±1°C. After 15 minutes, the flasks were removed and 33 mL of the cell suspension was pipetted into each of three appropriately labeled petri dishes. To accelerate the gelling process, the plates were placed in cold storage (approximately 4°C) for approximately 30 minutes. The plates were then incubated at 37±1°C in a humidified 5±1% CO_2 atmosphere for 10-14 days.

Scoring Procedures

After the incubation period, the V.C. plates were counted for the total number of colonies per plate and the total relative growth determined. The TFT-resistant colonies were then counted for each culture with 10% total relative growth. The diameters of the TFT-resistant colonies for the positive and solvent controls and, in the case of a positive response, the test article-treated cultures were determined over a range of approximately 0.2 to 1.1 mm. The rationale for this procedure is as follows: Mutant L5178Y $TK^{-/-}$ colonies exhibit a characteristic frequency distribution of colony sizes. The precise distribution of large and small TFT-resistant mutant colonies appears to be the characteristic mutagenic "finger-print" of carcinogens in the L5178Y $TK^{+/-}$ system (Clive *et al.*, 1979; DeMarini *et al.*, 1989). Clive *et al.* (1979) and Hozier *et al.* (1981) have presented evidence to substantiate the hypothesis that the small colony variants carry chromosome aberrations associated with chromosome 11, the chromosome on which the TK locus is located in the mouse (Kozak and Ruddle, 1977). They suggested that large colony mutants received very localized damage, possibly in the form of a point mutation or small deletion within the TK locus, while small colony mutants received damage to collateral loci concordant with the loss of TK activity.

Evaluation of Results

The cytotoxic effects of each treatment condition were expressed relative to the solvent-treated control for suspension growth over two days post-treatment and for total growth (suspension growth corrected for plating efficiency at the time of selection). The mutant frequency (number of mutants per 10^6 surviving cells) was determined by dividing the average number of colonies in the three TFT plates by the average number of colonies in the three corresponding V.C. plates and multiplying by the dilution factor ($2x10^{-4}$) then multiplying by 10^6. For simplicity, this is described as: (Average # TFT colonies / average # VC colonies) x 200 in the tables.

In evaluation of the data, increases in mutant frequencies that occurred only at highly toxic concentrations (i.e., less than 10% total growth) were not considered biologically relevant. All conclusions were based on sound scientific judgement; however, the following criteria are presented as a guide to interpretation of the data (Clive *et al.*, 1995):

- The result was considered to induce a positive response if a concentration-related increase in mutant frequency was observed and one or more dose levels with 10% or greater total growth exhibited mutant frequencies of 100 mutants per 10^6 clonable cells over the background level.

- A result was considered equivocal if the mutant frequency in treated cultures was between 55 and 99 mutants per 10^6 clonable cells over the background level.

- Test articles producing fewer than 55 mutants per 10^6 clonable cells over the background level were concluded to be negative.

Criteria for a Valid Test

The following criteria must be met for the mutagenesis assay to be considered valid:

Negative Controls:

The spontaneous mutant frequency of the solvent control cultures must be within 20 to 100 TFT-resistant mutants per 10^6 surviving cells. The cloning efficiency of the solvent control group must be greater than 50%.

Positive Controls:

At least one concentration of each positive control must exhibit mutant frequencies of 100 mutants per 10^6 clonable cells over the background level. The colony size distribution for the MMS positive control must show an increase in both small and large colonies (Moore *et al.*, 1985; Aaron *et al.*, 1994).

Test Article-Treated Cultures:

A minimum of four analyzable concentrations with mutant frequency data will be required.

Archives

All raw data, protocol, and a copy of all reports will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance RAQA unit headquartered at:

BioReliance
14920 Broschart Rd.
Rockville, MD 20850

RESULTS AND DISCUSSION

Solubility Test

Dimethyl sulfoxide (DMSO) was selected as the solvent of choice based on solubility of the test article and compatibility with the target cells. The test article was soluble in DMSO at 500 mg/mL, the maximum concentration tested.

Preliminary Toxicity Assay

The results of the preliminary toxicity assay are presented in Table 1. The maximum dose tested in the preliminary toxicity assay was 5000 μg/mL. No visible precipitate was present at any dose level in treatment medium. The osmolality of the solvent control was 447 mmol/kg and the osmolality of the highest soluble dose, 5000 μg/mL, was 462 mmol/kg. Suspension growth relative to the solvent controls was 89% at 5000 μg/mL without activation and 72% at 5000 μg/mL with S9 activation. Based on the results of the toxicity test, the doses chosen for the mutagenesis assay ranged from 50 to 5000 μg/mL for both the non-activated and S9-activated cultures.

Mutagenesis Assay

The results of the mutagenesis assay are presented in Tables 2 through 5. Colony size distributions are presented in Figures 1 and 2. No visible precipitate was present at any dose level in treatment medium. In the non-activated system, cultures treated with concentrations of 1000, 2000, 3000, 4000 and 5000 μg/mL were cloned and produced a range in suspension growth of 61% to 98%. In the S9-activated system, cultures treated with concentrations of 1000, 2000, 3000, 4000 and 5000 μg/mL were cloned and produced a range in suspension growth of 14% to 80%.

No cloned cultures exhibited mutant frequencies that were at least 55 mutants per 10^6 clonable cells over that of the solvent control. A dose-response trend was not observed in the non-activated or S9-activated systems. The total growths ranged from 69% to 92% for the non-activated cultures at concentrations of 1000 to 5000 μg/mL and 13% to 85% for the S9-activated cultures at concentrations of 1000 to 5000 μg/mL.

The TFT-resistant colonies for the positive and solvent control cultures were sized according to diameter over a range from approximately 0.2 to 1.1 mm. The colony sizing for the MMS positive control yielded the expected increase in small colonies, verifying the adequacy of the methods used to detect small colony mutants.

CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the L5178Y/TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay indicate that, under the conditions of this study, ammonium perchlorate was concluded to be negative.

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TABLE 1

PRELIMINARY TOXICITY ASSAY USING ammonium perchlorate

Test Article Concentration (µg/mL)	Cell Concentration (X10^6)[a] Day 1	Day 2	Suspension Growth % of Total[b]	Control[c]
WITHOUT ACTIVATION				
Solvent 1	0.921	1.343	13.7	
Solvent 2	0.915	1.297	13.2	
.5	0.901	1.362	13.6	101
1.5	0.923	1.357	13.9	103
5	0.863	1.373	13.2	98
15	0.827	1.392	12.8	95
50	0.872	1.323	12.8	95
150	0.926	1.282	13.2	98
500	0.862	1.359	13.0	97
1500	0.895	1.281	12.7	95
5000	0.732	1.469	11.9	89
WITH S-9 ACTIVATION				
Solvent 1	0.663	1.292	9.5	
Solvent 2	0.650	1.333	9.6	
.5	0.686	1.368	10.4	109
1.5	0.693	1.379	10.6	111
5	0.700	1.349	10.5	110
15	0.652	1.307	9.5	99
50	0.663	1.341	9.9	103
150	0.647	1.316	9.5	99
500	0.661	1.333	9.8	102
1500	0.606	1.413	9.5	99
5000	0.507	1.224	6.9	72

Solvent = DMSO

1 and 2 are duplicate cultures

[a] - Cultures containing $<0.3 \times 10^6$ cells/mL on day 1 and 2 are considered as having 0% total suspension growth.

[b] - Total suspension growth = (Day 1 cell conc. / 0.3×10^6 cells/mL) x (Day 2 cell conc. / Day 1 adjusted cell conc.)

[c] - % of control suspension growth = (total treatment suspension growth / average solvent control total suspension growth) x 100

TABLE 2

CLONING DATA FOR L5178Y/TK$^{+/-}$ MOUSE LYMPHOMA CELLS TREATED WITH ammonium perchlorate IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Test Article Concentration (µg/mL)		TFT Colonies Counts			Mean	VC Colonies Counts				Mean	Mutant Freq.[a]	Induced Mutant Freq.[b]	% Total Growth[c]
Solvent	1	22	21	24	22 ±1	191	178	170	180	±9	25		
Solvent	2	20	17	25	21 ±3	179	140	172	164	±17	25		
Mean Solvent Mutant Frequency= 25													
1000	A	27	17	15	20 ±5	149	176	148	158	±13	25	0	83
1000	B	13	21	24	19 ±5	157	156	131	148	±12	26	1	84
2000	A	28	26	14	23 ±6	168	155	159	161	±5	28	3	81
2000	B	18	22	19	20 ±2	172	181	169	174	±5	23	-2	92
3000	A	25	25	25	25 ±0	172	158	163	164	±6	30	5	90
3000	B	24	24	26	25 ±1	153	177	181	170	±12	29	4	85
4000	A	19	14	14	16 ±2	190	179	197	189	±7	17	-8	86
4000	B	31	20	24	25 ±5	205	184	207	199	±10	25	0	92
5000	A	17	24	25	22 ±4	195	183	157	178	±16	25	0	69
5000	B	24	34	32	30 ±4	203	188	189	193	±7	31	6	69
Positive Control - Methyl Methanesulfonate (µg/mL)													
10		106	139	159	135 ±22	114	110	114	113	±2	239	214	48
20		106	127	119	117 ±9	35	40	46	40	±4	582	557	12

Solvent = DMSO

A and B or 1 and 2 are duplicate cultures

[a] - Mutant frequency (per 10^6 surviving cells)=(Average # TFT colonies / average # VC colonies) x 200

[b] - Induced mutant frequency (per 10^6 surviving cells) = mutant frequency - average mutant frequency of solvent controls

[c] - % total growth = (% suspension growth x % cloning growth) / 100

TABLE 3

TOTAL COMPOUND TOXICITY DATA FOR L5178Y/TK$^{+/-}$ MOUSE LYMPHOMA CELLS TREATED WITH ammonium perchlorate IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Test Article Concentration (µg/mL)		Cell Concentration (X 10^6)[a] Day 1	Day 2	Susp Growth Total[b]	%Cntl[c]	Cloning Growth Avg VC	%Cntl[d]	% Total Growth[e]
Solvent	1	1.438	1.362	21.8		180		
Solvent	2	1.446	1.270	20.4		164		
1000	A	1.229	1.397	19.1	90	158	92	83
1000	B	1.301	1.425	20.6	98	148	86	84
2000	A	1.236	1.333	18.3	87	161	94	81
2000	B	1.311	1.306	19.0	90	174	101	92
3000	A	1.200	1.492	19.9	94	164	96	90
3000	B	1.187	1.367	18.0	85	170	99	85
4000	A	1.131	1.318	16.6	79	189	110	86
4000	B	1.135	1.323	16.7	79	199	116	92
5000	A	1.062	1.193	14.1	67	178	104	69
5000	B	1.068	1.085	12.9	61	193	113	69
Positive Control - Methyl Methanesulfonate (µg/mL)								
10		1.232	1.134	15.5	74	113	66	48
20		1.081	0.884	10.6	50	40	23	12

Solvent = DMSO

A and B or 1 and 2 are duplicate cultures

[a] - Cultures containing <0.3x10^6 cells/mL on day 1 and 2 are considered as having 0% total suspension growth.

[b] - Total suspension growth = (Day 1 cell conc. / 0.3x10^6 cells/mL) x (Day 2 cell conc. / Day 1 adjusted cell conc.)

[c] - % of control suspension growth = (total treatment suspension growth / average solvent control total suspension growth) x 100

[d] - % control cloning growth = (average V.C. of treated culture / average V.C. of solvent control) x 100

[e] - % total growth = (% suspension growth x % cloning growth) / 100

TABLE 4

CLONING DATA FOR L5178Y/TK$^{+/-}$ MOUSE LYMPHOMA CELLS TREATED WITH ammonium perchlorate IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Test Article Concentration (µg/mL)		TFT Colonies Counts			Mean	VC Colonies Counts				Mean	Mutant Freq.[a]	Induced Mutant Freq.[b]	% Total Growth[c]
Solvent	1	21	28	30	26 ±4	150	130	157	146	±11	36		
Solvent	2	24	34	50	36 ±11	163	178	163	168	±7	43		
Mean Solvent Mutant Frequency= 40													
1000	A	24	30	18	24 ±5	186	208	166	187	±17	26	-14	80
1000	B	15	19	28	21 ±5	158	157	181	165	±11	25	-15	85
2000	A	35	35	25	32 ±5	190	173	153	172	±15	37	-3	65
2000	B	21	24	26	24 ±2	199	170	207	192	±16	25	-15	82
3000	A	35	38	33	35 ±2	192	165	169	175	±12	40	1	58
3000	B	28	22	34	28 ±5	186	176	186	183	±5	31	-9	64
4000	A	38	32	33	34 ±3	169	172	150	164	±10	42	2	41
4000	B	33	28	34	32 ±3	183	185	175	181	±4	35	-5	42
5000	A	38	34	47	40 ±5	135	137	142	138	±3	57	18	13
5000	B	+	40	50	45 ±4	191	162	173	175	±12	51	12	21
Positive Control - 7,12 Dimethylbenz(a)anthracene (µg/mL)													
2.5		135	129	136	133 ±3	120	129	137	129	±7	207	168	65
4		171	166	189	175 ±10	130	111	99	113	±13	309	270	42

Solvent = DMSO

A and B or 1 and 2 are duplicate cultures

+ - Culture lost to contamination

[a] - Mutant frequency (per 10^6 surviving cells)=(Average # TFT colonies / average # VC colonies) x 200

[b] - Induced mutant frequency (per 10^6 surviving cells) = mutant frequency - average mutant frequency of solvent controls

[c] - % total growth = (% suspension growth x % cloning growth) / 100

TABLE 5

TOTAL COMPOUND TOXICITY DATA FOR L5178Y/TK$^{+/-}$ MOUSE LYMPHOMA CELLS TREATED WITH ammonium perchlorate IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Test Article Concentration (µg/mL)		Cell Concentration (X 10^6)[a] Day 1	Day 2	Susp Growth Total[b]	%Cntl[c]	Cloning Growth Avg VC	%Cntl[d]	% Total Growth[e]
Solvent	1	1.291	1.443	20.7		146		
Solvent	2	1.315	1.503	22.0		168		
1000	A	1.150	1.129	14.4	68	187	119	80
1000	B	1.225	1.256	17.1	80	165	105	85
2000	A	1.014	1.114	12.5	59	172	110	65
2000	B	1.060	1.218	14.3	67	192	122	82
3000	A	0.919	1.088	11.1	52	175	112	58
3000	B	0.845	1.248	11.7	55	183	116	64
4000	A	0.685	1.108	8.4	40	164	104	41
4000	B	0.642	1.083	7.7	36	181	115	42
5000	A	0.293	0.919	3.1	14	138	88	13
5000	B	0.412	0.866	4.0	19	175	112	21
Positive Control - 7,12 Dimethylbenz(a)anthracene (µg/mL)								
2.5		1.087	1.397	16.9	79	129	82	65
4		0.949	1.188	12.5	59	113	72	42

Solvent = DMSO

A and B or 1 and 2 are duplicate cultures

[a] - Cultures containing <0.3x10^6 cells/mL on day 1 and 2 are considered as having 0% total suspension growth.

[b] - Total suspension growth = (Day 1 cell conc. / 0.3x10^6 cells/mL) x (Day 2 cell conc. / Day 1 adjusted cell conc.)

[c] - % of control suspension growth = (total treatment suspension growth / average solvent control total suspension growth) x 100

[d] - % control cloning growth = (average V.C. of treated culture / average V.C. of solvent control) x 100

[e] - % total growth = (% suspension growth x % cloning growth) / 100

Figure 1

Colony Size Distribution in the Absence of Metabolic Activation

(Positive Control Compared with Solvent Control)

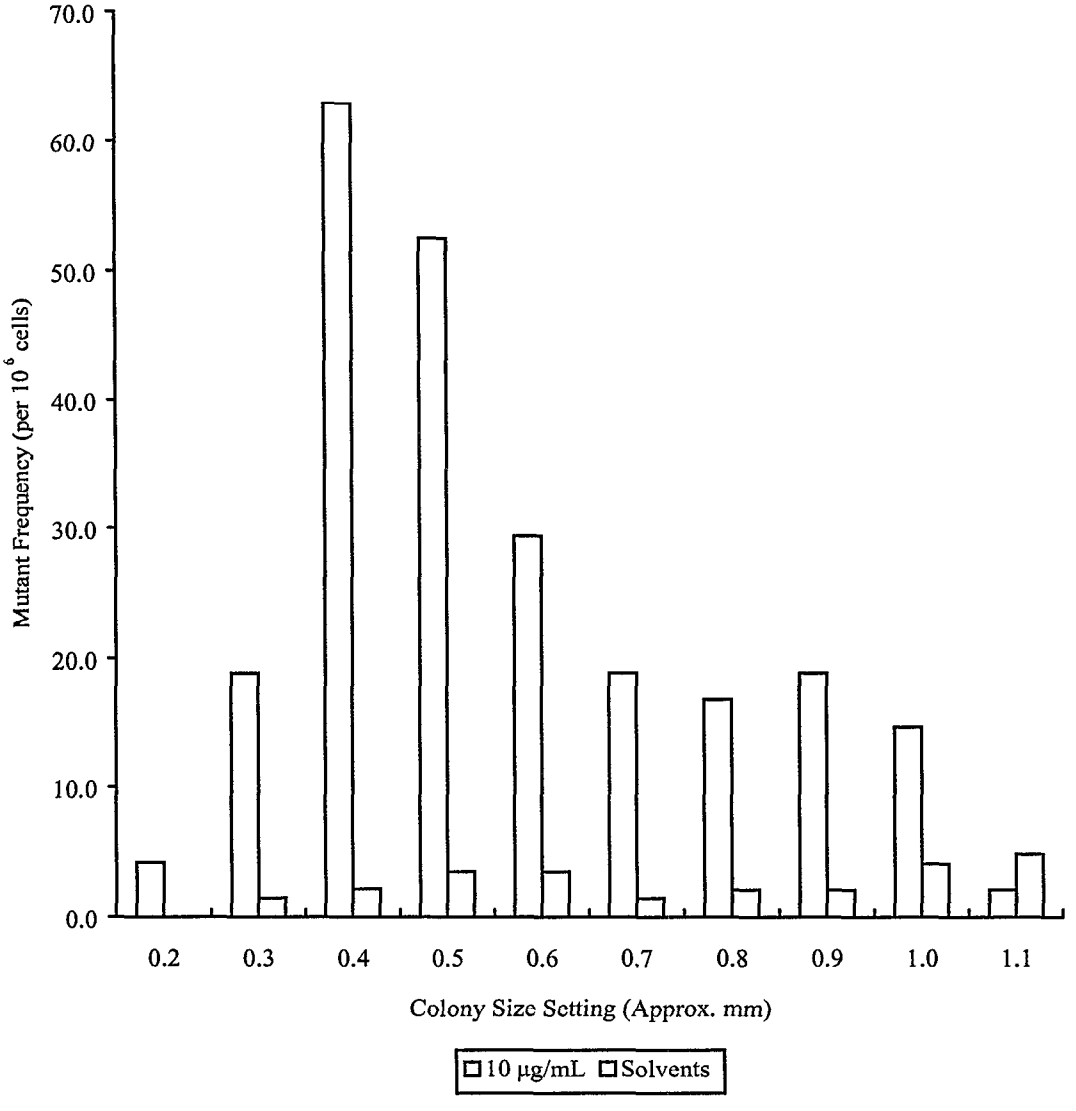
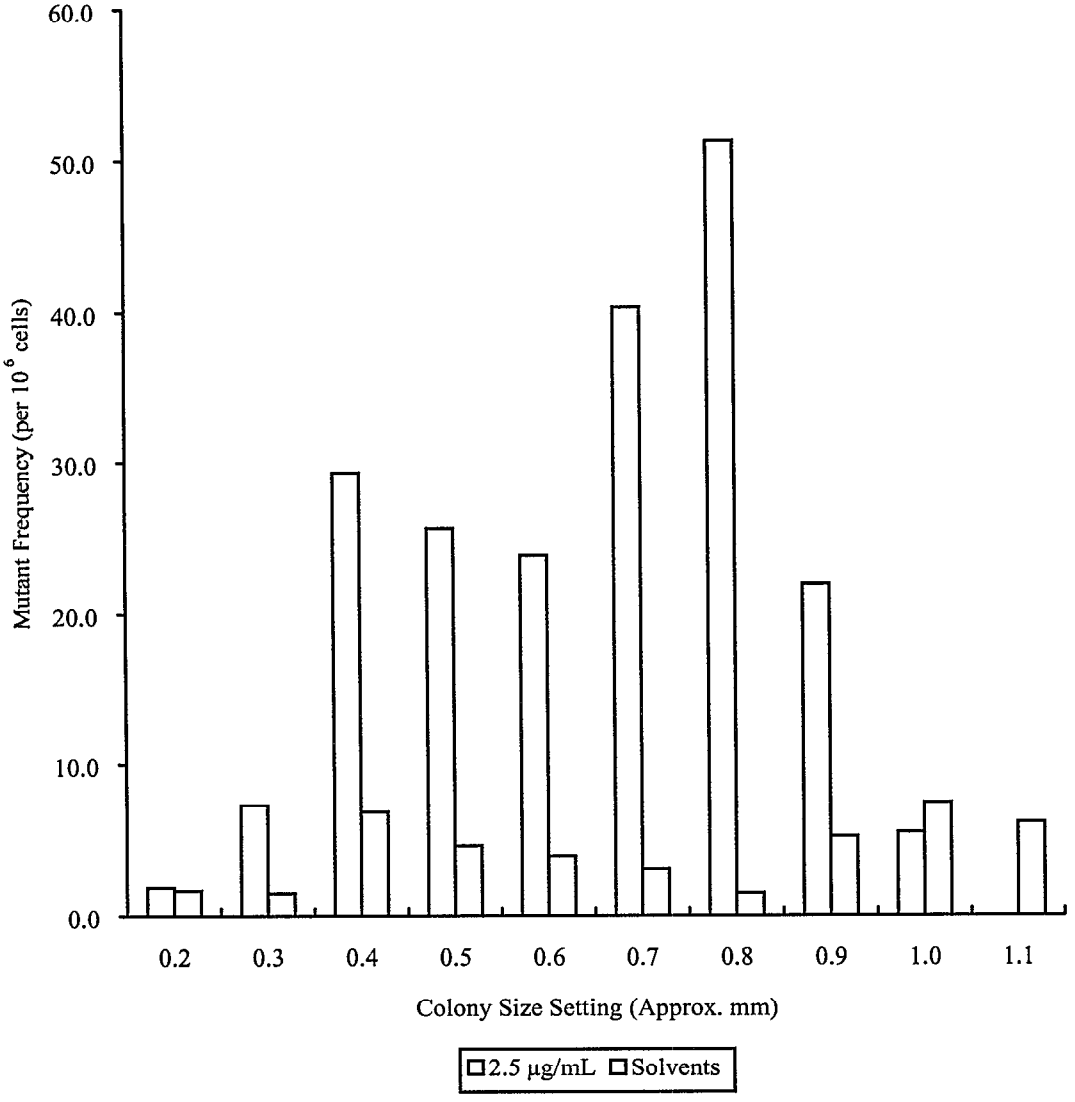


Figure 2

Colony Size Distribution in the Presence of Metabolic Activation

(Positive Control Compared with Solvent Control)



APPENDIX I

Historical Control Data

Mouse Lymphoma Historical Control Data

1995-1997

	Non-activated			S9-Activated		
	Solvent Control	20 •g/mL MMS	10 •g/mL MMS	Solvent Control	4.0•g/mL DMBA	2.5•g/mL DMBA
Mean MF	35.7	655.3	336.0	58.0	453.2	269.8
SD	10.3	293.3	128.5	18.6	158.5	95.1
Maximum	68.0	2400.0	729.0	100.0	1029.0	1048.0
Minimum	20.0	198.0	128.0	28.0	222.0	141.0

Solvent control (Fischer's medium, distilled water, saline, DMSO, ethanol, acetone or vehicle supplied by Sponsor)

MMS Methyl methanesulfonate
DMBA Dimethylbenz(a)anthracene
MF Mutant frequency per 10^6 clonable cells
SD Standard deviation

APPENDIX II

Study Protocol

QA 12-4-98
APPROVED

RECEIVED BY RADIA 12/02/98
BioReliance Study Number: G98BA06.702

In Vitro Mammalian Cell Gene Mutation Test (L5178Y/TK$^{+/-}$ Mouse Lymphoma Assay)

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article based on quantitation of forward mutations at the thymidine kinase locus of L5178Y mouse lymphoma cells.

2.0 SPONSOR

2.1	Name:	**Perchlorate Study Group**
2.2	Address:	Highway 50 and Aerojet Road Building 20019/Department 0330 Rancho Cordova, CA 95813-6000
2.3	Study Monitor:	Michael F. Girard Perchlorate Study Group Representative Telephone: (916) 355-2945 Telefax: (916) 355-6145
2.4	Scientific Advisor:	Michael L. Dourson, Ph.D., DABT Toxicology Excellence for Risk Assessment 4303 Hamilton Ave. Cincinnati, OH 45223 Telephone: (513) 542-7475 Telefax: (513) 542-7487
2.5	Sponsor Project #:	NP

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1	Test Article:		**Ammonium perchlorate**
3.2	Controls:	Negative:	Test article solvent (or vehicle)
		Positive:	Methyl methanesulfonate (MMS) 7,12-dimethylbenz(a)anthracene (DMBA)

3.3 Determination of Strength, Purity, etc.

Unless alternate arrangements are made, the testing facility at BioReliance will not perform analysis of the dosing solutions. The Sponsor will be directly responsible

for determination and documentation of the analytical purity and composition of the test article, and the stability and strength of the test article in the solvent (or vehicle).

3.4 Test Article Retention Sample

The retention of a reserve sample of the test article will be the responsibility of the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1	Name:	Toxicology Testing Facility BioReliance
4.2	Address:	9630 Medical Center Drive Rockville, MD 20850
4.3	Study Director:	Richard H. C. San, Ph.D.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 12/7/98

5.2 Proposed Experimental Completion Date: 1/19/99

5.3 Proposed Report Date: 1/28/99

6.0 TEST SYSTEM

L5178Y/TK$^{+/-}$ mouse lymphoma cells are heterozygous at the normally diploid thymidine kinase (TK) locus. L5178Y/TK$^{+/-}$, clone 3.7.2C, were received from Patricia Poorman-Allen, Glaxo Wellcome Inc., Research Triangle Park, North Carolina. Each freeze lot of cells has been tested and found to be free of mycoplasma contamination. This system has been demonstrated to be sensitive to the mutagenic activity of a variety of chemicals.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The mammalian mutation assay will be performed by exposing duplicate cultures of L5178Y/TK$^{+/-}$ cells to a minimum of eight concentrations of test article as well as positive and negative (solvent) controls. Exposures will be for 4 hours in the presence and absence of an S9 activation system. Following a two day expression period, with daily cell population adjustments, cultures demonstrating 0% to 90% growth inhibition will be cloned, in triplicate, in restrictive medium containing soft agar to select for the mutant phenotype. After a 10 to 14 day selection period, mutant colonies will be enumerated. The mutagenic potential of the test article will be measured by its ability to induce TK$^{+/-} \rightarrow$ TK$^{-/-}$ mutations. For those test articles demonstrating a positive response, mutant colonies will be sized as an indication of mechanism of action.

7.1 Selection of Solvent

Unless the Sponsor has indicated the test article solvent, a solubility determination will be conducted to measure the maximum soluble concentration in a variety of solvents. Solvents compatible with this test system, in order of preference, include, but are not limited to, culture medium or distilled water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone CAS 67-64-1). The solvent of choice will be that solvent, selected in order of preference, that permits preparation of the highest soluble stock concentration, up to a maximum of 500 mg/ml.

7.2 Dose Selection

In the preliminary toxicity test, L5178Y/TK$^{+/-}$ cells will be exposed to solvent alone and to at least nine concentrations of test article, the highest concentration being the lowest insoluble dose in treatment medium but not to exceed 5000 µg/ml. The pH of the treatment medium will be adjusted, if necessary, to maintain a neutral pH in the treatment medium. The osmolality of the highest soluble treatment condition will also be measured. After a 4-hour treatment in the presence and absence of S9 activation, cells will be washed twice with F_0P (Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronics) or $F_{10}P$ (F_0P supplemented with 10% horse serum and 2mM L-glutamine) and cultured in suspension for two days post-treatment, with cell concentration adjustment on the first day.

Selection of dose levels for the mutation assay will be based on reduction of suspension growth after treatment in the preliminary toxicity test. Unless specified otherwise by the Sponsor, the high dose for the mutation assay will be that concentration exhibiting approximately 100% growth inhibition. The low dose will be selected to exhibit 0% growth inhibition. For freely soluble, non-toxic test articles, the highest concentration will be 5000 µg/ml. For relatively insoluble, non-toxic test articles, the highest concentration will be the lowest insoluble dose in treatment medium but not to exceed 5000 µg/ml. In all cases, precipitation will be evaluated at the beginning and at the end of the treatment period using the naked eye (ICH, 1996).

7.3 Route and Frequency of Administration

Cell cultures will be treated for 4 hours by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This technique of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

7.4 Exogenous Metabolic Activation

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The source of S9 will be adult male Sprague-Dawley rats induced by a single injection of Aroclor 1254 at a dose level of 500 mg/kg body weight five days prior

to sacrifice. The S9 will be batch prepared and stored frozen at approximately -70°C until used.

Immediately prior to use, the S9 will be thawed and mixed with a cofactor pool to contain 11.25 mg DL-isocitric acid, 6 mg NADP, and 0.25 ml S9 homogenate per ml in F_0P. The S9 mix will be adjusted to pH 7.

7.5 Controls

7.5.1 Negative Control

The solvent (or vehicle) for the test article will be used as the negative control.

7.5.2 Positive Controls

Methyl methanesulfonate (MMS) will be used at two concentrations of 10 and 20 µg/ml as the positive control for the non-activated test system. For the S9-activated system, 7,12-dimethylbenz(a)anthracene (DMBA) will be used at two concentrations of 2.5 and 4.0 µg/ml.

7.6 Preparation of Target Cells

Prior to use in the assay, L5178Y/TK$^{+/-}$ cells will be cleansed to reduce the frequency of spontaneously occurring TK$^{-/-}$ cells. Using the procedure described by Clive and Spector (1975), L5178Y cells will be cultured for 24 hours in the presence of thymidine, hypoxanthine, methotrexate and glycine to poison the TK$^{-/-}$ cells.

L5178Y/TK$^{+/-}$ cells will be prepared at 1 x 10^6 cells/ml in 50% conditioned $F_{10}P$ and 50% F_0P. If cultures are to be treated with more than 100 µl of test article dosing solution, the cell concentration may be adjusted.

7.7 Identification of the Test System

Using a permanent marking pen, the treatment tubes will be identified by the study number and a code system to designate the treatment condition and test phase.

7.8 Treatment of Target Cells

Treatment will be carried out in conical tubes by combining 100 µl dosing solution of test or control article in solvent or solvent alone, 4 ml F_0P medium or S9 activation mixture with 6 x 10^6 L5178Y/TK$^{+/-}$ cells in a total volume of 10 ml. A minimum of eight concentrations of test article will be tested in duplicate. All pH adjustments will be performed prior to adding S9 or target cells to the treatment medium. Volumes of test article dosing solution in excess of 100 µl may be used if required to achieve the target final concentration in treatment medium. Treatment

tubes will be gassed with 5±1% CO_2 in air, capped tightly, and incubated with mechanical mixing for 4 hours at 37±1°C. The preparation and addition of the test article dosing solutions will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

7.9 Expression of the Mutant Phenotype

At the end of the exposure period, the cells will be washed twice with F_0P or $F_{10}P$ and collected by centrifugation. The cells will be resuspended in 20 ml $F_{10}P$, gassed with 5±1% CO_2 in air and cultured in suspension at 37±1°C for two days following treatment. Cell population adjustments to 0.3 x 10^6 cells/ml will be made at 24 and 48 hours.

7.10 Selection of the Mutant Phenotype

For selection of the trifluorothymidine (TFT)-resistant phenotype, cells from a minimum of five non-activated and five S9-activated test article concentrations demonstrating from 0% to 90% suspension growth inhibition will be plated into three replicate dishes at a density of 1 x 10^6 cells/100mm plate in cloning medium containing 0.23% agar and 2-4 µg TFT/ml. For estimation of cloning efficiency at the time of selection, 200 cells/100mm plate will be plated in triplicate in cloning medium free of TFT (viable cell (VC) plate). Plates will be incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 for 10-14 days.

The total number of colonies per plate will be determined for the VC plates and the total relative growth calculated. The total number of colonies per TFT plate will then be determined for those cultures with ≥10% total growth. Colonies are enumerated using an automatic counter; if the automatic counter cannot be used, the colonies will be counted manually. The diameters of the TFT colonies from the positive control and solvent control cultures will be determined over a range of approximately 0.2 to 1.1 mm. In the event the test article demonstrates a positive response, the diameters of the TFT colonies for at least one dose level of the test article (the highest positive concentration) will be determined over a range of approximately 0.2 to 1.1 mm.

7.11 Independent Repeat Assay

Verification of a clear positive response will not be required (OECD Guideline 476, ICH 1997). For equivocal and negative results, the Sponsor will be consulted regarding the need for an independent repeat assay.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Negative Controls

The spontaneous mutant frequency of the solvent (or vehicle) control cultures must be within 20 to 100 TFT-resistant mutants per 10^6 surviving cells. The cloning efficiency of the solvent (or vehicle) control group must be greater than 50%.

8.2 Positive Controls

At least one concentration of each positive control must exhibit mutant frequencies of ≥100 mutants per 10^6 clonable cells over the background level. The colony size distribution for the MMS positive control must show an increase in both small and large colonies (Moore *et al.*, 1985; Aaron *et al.*, 1994).

8.3 Test Article-Treated Cultures

A minimum of four analyzable concentrations with mutant frequency data will be required.

9.0 EVALUATION OF TEST RESULTS

The cytotoxic effects of each treatment condition are expressed relative to the solvent-treated control for suspension growth over two days post-treatment and for total growth (suspension growth corrected for plating efficiency at the time of selection). The mutant frequency for each treatment condition is calculated by dividing the mean number of colonies on the TFT-plates by the mean number of colonies on the VC-plates and multiplying by the dilution factor (2×10^{-4}), and is expressed as TFT-resistant mutants per 10^6 surviving cells.

In evaluation of the data, increases in mutant frequencies which occur only at highly toxic concentrations (i.e., less than 10% total growth) are not considered biologically relevant. All conclusions will be based on sound scientific judgement; however, the following criteria are presented as a guide to interpretation of the data (Clive *et al.*, 1995):

- The result will be considered to induce a positive response if a concentration-related increase in mutant frequency is observed and one or more dose levels with 10% or greater total growth exhibit mutant frequencies of ≥100 mutants per 10^6 clonable cells over the background level.

- A result will be considered equivocal if the mutant frequency in treated cultures is between 55 and 99 mutants per 10^6 clonable cells over the background level.

- Test articles producing fewer than 55 mutants per 10^6 clonable cells over the background level will be concluded to be negative.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used in the generation and analysis of data.

Results presented will include, but not be limited to:

- test substance: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of test article, if known.
- solvent/vehicle: justification for choice of vehicle; solubility and stability of test article in solvent/vehicle, if known.
- cell type used, number of cultures, methods for maintenance of cell cultures
- rationale for selection of concentrations and number of cultures
- test conditions: composition of media, CO_2 concentration, concentration of test substance, vehicle, incubation temperature, incubation time, duration of treatment, cell density during treatment, type of metabolic activation system, positive and negative controls, length of expression period, selective agent
- method used to enumerate numbers of viable and mutant colonies and the number of colonies in each plate
- dose-response relationship, if applicable
- distribution of the mutant colony diameter for the solvent and positive controls and, when the test article induces a positive response, for at least one dose level of the test article (the highest positive concentration)
- positive and solvent control historical data

11.0 RECORDS AND ARCHIVES

Upon completion of the final report, all raw data and reports will be maintained in the archives of BioReliance, Rockville, MD in accordance with the relevant Good Laboratory Practice Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with OECD Guideline for the Testing of Chemicals, Guideline 476 (*In Vitro* Mammalian Cell Gene Mutation Test), July 1997, and with the International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use, Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals, S2A document recommended for

adoption at step 4 of the ICH process on July 19, 1995. Federal Register 61:18198-18202. April 24, 1996.

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? yes

If so, to which agency or agencies? U.S. EPA, U.S. DOD

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test article will be disposed of following finalization of the report.

13.0 REFERENCES

Aaron, C.S., Bolcsfoldi, G., Glatt, H.-R., Moore, M., Nishi, Y., Stankowski, L., Theiss, J. and Thompson, E. (1994) Mammalian cell gene mutation assays working group report. Mutation Research 312:235-239.

Clive, D., Bolcsfoldi, G., Clements, J., Cole, J., Homna, M., Majeska, J., Moore, M., Muller, L., Myhr, B., Oberly, T., Oudelhkim, M., Rudd, C., Shimada, H., Sofuni, T., Thybaud, V. and Wilcox, P. (1995) Consensus agreement regarding protocol issues discussed during the mouse lymphoma workshop: Portland, Oregon, May 7, 1994. Environ. Molec. Mutagen. 25:165-168.

Clive, D. and Spector, J.F.S. (1975) Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutation Research 31:17-29.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. S2A document recommended for adoption at step 4 of the ICH process on July 19, 1995. Federal Register 61:18198-18202. April 24, 1996.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals. S2B document recommended for adoption at step 4 of the ICH process on July 16, 1997. Federal Register 62:16026-16030, November 21, 1997.

Moore, M.M., Clive, D., Howard, B.E., Batson, A.G. and Turner, N.T. In situ analysis of trifluorothymidine-resistant (TFT^r) mutants of L5178Y/TK$^{+/-}$ mouse lymphoma cells. (1985) Mutation Research 151:147-159.

OECD Guideline for the Testing of Chemicals, Guideline 476 (*In Vitro* Mammalian Cell Gene Mutation Test), July 1997.

14.0 APPROVAL

Michael F. Girard — 11/19/98
Sponsor Study Monitor — Date

Michael L. Dourson, Ph.D., DABT — 11:10:98
Sponsor Scientific Advisor — Date

Richard H.C. San, Ph.D. — 12/2/98
BioReliance Study Director — Date

If submission to Japanese Regulatory Agency is indicated in section 12.0, BioReliance management will sign.

David Jacobson-Kram, Ph.D., DABT — Date
BioReliance Study Management